



Control of cell proliferation in atherosclerosis: insights from animal models and human studies

José J. Fuster¹, Patricia Fernández¹, Herminia González-Navarro¹,
Carlos Silvestre^{1,2}, Yafa Naim Abu Nabah¹, and Vicente Andrés^{1,2*}

¹Laboratory of Vascular Biology, Department of Molecular and Cellular Pathology and Therapy, Instituto de Biomedicina de Valencia-CSIC, C/Jaime Roig 11, 46010 Valencia, Spain; and ²Laboratory of Molecular and Genetic Cardiovascular Pathophysiology, Department of Atherothrombosis and Cardiovascular Imaging, Spanish National Cardiovascular Research Center (CNIC), Melchor Fernández Almagro 3, 28029 Madrid, Spain

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Excessive hyperplastic cell growth within occlusive vascular lesions has been recognized as a key component of the inflammatory response associated with atherosclerosis, restenosis post-angioplasty, and graft atherosclerosis after coronary artery bypass. Understanding the molecular mechanisms that regulate arterial cell proliferation is therefore essential for the development of new tools for the treatment of these diseases. Mammalian cell proliferation is controlled by a large number of proteins that modulate the mitotic cell cycle, including cyclin-dependent kinases, cyclins, and tumour suppressors. The purpose of this review is to summarize current knowledge about the role of these cell cycle regulators in the development of native and graft atherosclerosis that has arisen from animal studies, histological examination of specimens from human patients, and genetic studies.

Keywords Cell cycle • Human atherosclerosis • Animal models atherosclerosis

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1. Introduction

Atherosclerosis, restenosis (vessel renarrowing after successful angioplasty), and graft atherosclerosis after coronary artery bypass surgery are chronic inflammatory diseases that lead to the formation of obstructive vascular lesions. Excessive cell proliferation within the arterial wall is a key contributor to plaque growth; therefore, understanding the molecular mechanisms that control hyperplastic growth of vascular cells is of utmost importance for the development of efficient therapies against atherosclerosis and restenosis.

Mammalian cell proliferation is controlled by a large number of proteins that modulate the mitotic cell cycle (Figure 1). Progression through the cell cycle requires the activation of holoenzymes composed of a catalytic cyclin-dependent protein kinase (CDK) and the regulatory subunit cyclin. Specific CDKs are sequentially activated during different phases of the cell cycle by the oscillating synthesis and degradation of their cyclin partners. The activity of CDK/cyclins is also regulated by phosphorylation/dephosphorylation cycles and by their interaction with

CDK inhibitory proteins (CKIs) of the Cip/Kip (CDK interacting protein/kinase inhibitory protein: p21^{Cip1}, p27^{Kip1}, p57^{Kip2}) and Ink4 (inhibitor of CDK4: p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, p19^{Ink4d}) families.¹ All Cip/Kip proteins bind to and inhibit a wide spectrum of CDK/cyclin complexes, while the Ink4 proteins specifically inhibit cyclin D-associated CDKs. Mitogenic and antimitogenic stimuli affect the rates of CKI synthesis and degradation, as well as their redistribution among different CDK/cyclin heterodimers. In addition, other proteins, such as the transcriptional regulator p53, modulate the expression and function of CKIs to ensure that cells do not progress to the next phase of the cell cycle before appropriate conditions have been reached. CDK/cyclin activity modulates E2F/DP- and retinoblastoma protein (Rb)-dependent transcription of target genes involved in cell cycle control and DNA biosynthesis (Figure 2). In non-proliferating cells, lack of CDK/cyclin activity leads to the accumulation of hypophosphorylated Rb, which binds to and inactivates the dimeric transcription factor E2F/DP. In proliferating cells, CDK/cyclin activation causes the accumulation of hyperphosphorylated Rb during late G1-phase, thus causing the release of E2F/DP and

* Corresponding author. Tel: +34 914531200, Fax: +34 914531265, Email: vandres@cnic.es

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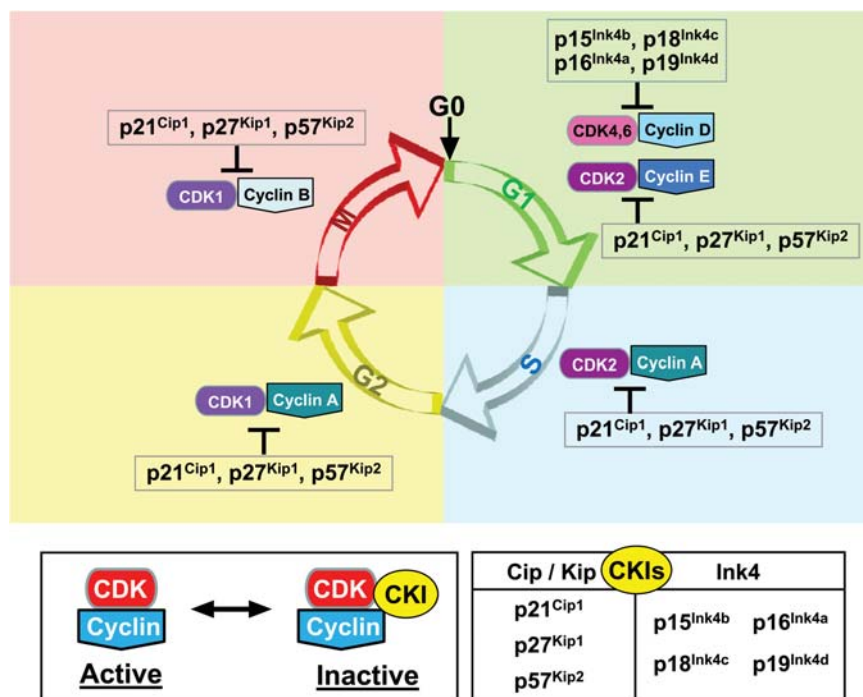


Figure 1 Mammalian cell cycle regulation by CDK/cyclin holoenzymes and CKIs. The cell cycle consists of four distinct phases: G₁, S (DNA replication), G₂, and M (mitosis). Activation of specific CDK/cyclin complexes drives progression through these cell cycle phases. CKIs of the Cip/kip and the INK4 families interact with and inactivate CDK/cyclin holoenzymes, thereby blocking cell cycle progression and cell proliferation.

the transactivation of various target genes necessary for cell cycle progression.

Cellular proliferation within atherosclerotic and restenotic plaques has been predominantly observed in vascular smooth muscle cells (VSMCs) and macrophages. Animal studies and tissue culture experiments have demonstrated that several cardiovascular risk factors promote the proliferation of these cell types,^{2–4} and there is evidence that these risk factors affect the expression or activity of CDK/cyclins and CKIs. For example, the mitogenic effects of oxLDL and homocysteine on VSMCs have been associated with higher expression of several cyclins and increased activity of CDKs.^{5,6} Similarly, angiotensin II treatment increases cyclin D1 and CDK4 expression and decreases p21^{Cip1} and p27^{Kip1} levels in rat mesenteric arteries.⁷ This review discusses animal and human studies that examine the role of cell cycle regulators in native and graft atherosclerosis. Although these proteins also play an important role in restenosis post-angioplasty, this is beyond the objectives of this article and is reviewed elsewhere.^{8,9}

2. Cell cycle regulators in experimental atherosclerosis

This section reviews animal studies assessing the role in atherosclerosis of various proteins involved in cell cycle regulation, including the transcriptional regulators p53 and Rb, and the Cip/

Kip family members p27^{Kip1} and p21^{Cip1} (summarized in Table 1). To date, no animal model studies have been reported on the role in atherosclerosis of p57^{Kip2}, p15^{INK4b}, p16^{INK4a}, p18^{INK4c} and p19^{INK4d}. However, such studies appear warranted, given the recent genome-wide association studies suggesting a role for the INK4/ARF locus in the development of coronary artery disease (CAD) and myocardial infarction (MI) (see below).

2.1 p53

The tumour suppressor p53 mediates the cellular response to a variety of stresses mainly by regulating the transcription of over 150 genes.¹⁰ Active p53 can induce reversible growth arrest in the G₁ or G₂ phases of the cell cycle, as well as cellular senescence or apoptosis. The first evidence supporting a role for p53 in the development of atherosclerosis was reported by Guevara *et al.*,¹¹ who demonstrated that p53 deficiency accelerates atheroma development in the aorta of apolipoprotein E (apoE)-null mice, coinciding with increased cell proliferation within the atheroma. Later, Mercer *et al.*¹² confirmed the enhanced aortic atherosclerosis of mice doubly deficient for p53 and apoE compared with apoE-null controls. However, in this latter study, p53 deficiency did not significantly increase atheroma development in the brachiocephalic artery, suggesting that the atheroprotective actions of p53 depends on the vascular bed being examined. Lesions in the brachiocephalic artery of p53-deficient animals contained an increased proportion of proliferating cells and reduced numbers of apoptotic

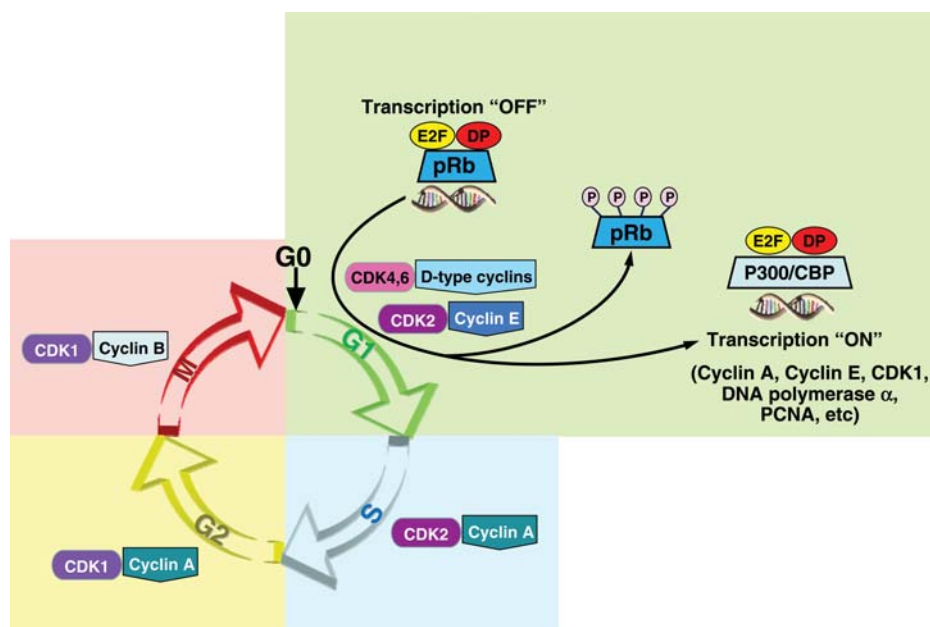


Figure 2 Cell cycle control by E2F/DP and Rb. In G1, in the presence of low level of CKIs, active CDK/cyclin complexes trigger the hyperphosphorylation of Rb, releasing the dimeric transcription factor E2F/DP and thereby inducing the transactivation of genes with functional E2F-binding sites (for example, growth and cell cycle regulators and genes encoding proteins required for nucleotide and DNA biosynthesis).

cells. Apoptosis within the atheroma affected both macrophages and VSMC, whereas most proliferating cells were monocytes/macrophages.¹²

The role of p53 in atherosclerosis development has also been assessed by using bone marrow transplantation (BMT) strategies to selectively inactivate p53 in haematopoietic cells. Van Vlijmen et al.¹³ reported that lethally irradiated apoE*3-Leiden mice transplanted with p53-deficient bone marrow show a significant increase in aortic atherosclerosis compared with mice transplanted with p53-wild-type marrow. This finding coincided with a non-significant tendency towards decreased apoptosis in mice receiving p53-deficient marrow, and the authors suggested this to be the mechanism underlying their findings. In contrast to previous studies of the effect of global inactivation of p53,^{11,12} cell proliferation within the atheroma was not affected by p53-deficient BMT.¹³ Merched et al.¹⁴ used a similar approach to analyse the effect of p53 haematopoietic inactivation on atheroma development in LDLR-null mice, another widely used model of atherosclerosis. These authors found that mice receiving p53-deficient BMT develop significantly larger aortic atheromas than mice transplanted with p53-wild-type bone marrow. However, in contrast to the results of Van Vlijmen et al.,¹³ p53-deficient BMT was associated with exacerbated cell proliferation within the atheroma.¹⁴ Using a different approach, Mercer et al.¹² found that transplantation of p53 wild-type bone marrow into p53-deficient apoE-null mice reduced aortic plaque formation and neointimal cell proliferation in brachiocephalic arteries, but also markedly reduced apoptosis. Very recently, Boesten et al.¹⁵ showed that atherosclerosis development in the aortic root, aortic arch, and thoracic aorta of fat-fed apoE-null mice is unaffected by Cre-loxP-mediated

macrophage-selective inactivation of p53. Cell proliferation within the atheroma was also unaffected by macrophage-specific p53 deficiency, whereas plaque apoptosis was markedly reduced.

Therefore, while most studies suggest an atheroprotective role for p53, it is unclear whether this is achieved mainly through effects on cell proliferation or apoptosis. The conflicting data among studies might result from experimental differences; for example, type of diet, periods of fat-feeding, and use of animal models with different genetic modifications. Alternatively, they might reflect cell-type-specific effects of p53, since p53-deficient peritoneal macrophages exhibit reduced apoptosis under standard culture conditions, whereas p53-deficient VSMCs exhibit increased apoptosis.¹² In this regard, it should be noted that adenovirus-mediated overexpression of p53 induces VSMC apoptosis in a collar model of murine atherosclerosis, suggesting that the antiapoptotic actions of p53 in VSMCs in culture might not occur in the atherosclerotic plaque.¹⁶ Another possible explanation is the different technologies used to achieve tissue-specific p53 inactivation, since BMT yields gene inactivation in both lymphoid and myeloid haematopoietic lineages, while the Cre-LoxP technology used by Boesten et al.¹⁵ inactivates the target gene only in myeloid cells. Moreover, BMT might also provoke gene inactivation in a subset of VSMCs and endothelial cells (ECs), since bone marrow progenitors can also differentiate into these cell types.^{17–19}

Although the underlying mechanism of p53's atheroprotective action remains undefined, strategies to increase its expression or function might be predicted to inhibit atherosclerosis. However, p53 gain-of-function studies performed in our laboratory appear to challenge this possibility, since atherosclerosis is unaffected in

Table 1 Atherosclerosis development after genetic manipulation of cell-cycle regulators in the mouse

Mouse model	Genetic modification	Effect on atheroma size	Effect on cell proliferation	Effect on apoptosis	Reference
apoE-null	p53 global inactivation	Increase	Increase	None	Guevara <i>et al.</i> ¹¹
apoE-null	p53 global inactivation	Increase (aorta); none (brachiocephalic)	Increase (brachiocephalic)	Reduction (brachiocephalic)	Mercer <i>et al.</i> ¹²
apoE-null	p53 ^{+/+} BMT in p53 ^{-/-} mice	Reduction (aorta); none (brachiocephalic)	Reduction (brachiocephalic)	Reduction (brachiocephalic)	Mercer <i>et al.</i> ¹²
apoE-null	Macrophage-specific p53 deficiency	None	None	Reduction	Boesten <i>et al.</i> ¹⁵
apoE*3-Leiden	p53 ^{-/-} BMT in p53 ^{+/+} mice	Increase	None	None	van Vlijmen <i>et al.</i> ¹³
LDLR-null	p53 ^{-/-} BMT in p53 ^{+/+} mice	Increase	Increase	None	Merched <i>et al.</i> ¹⁴
apoE-null	One extra p53 allele (normally regulated)	None	None	None	Sanz-González <i>et al.</i> ²⁰
apoE-null	Macrophage-specific Rb deficiency	Increase	Increase	None	Boesten <i>et al.</i> ²³
apoE-null	p27 ^{Kip1} global inactivation	Increase	Increase	Not studied	Díez-Juan and Andrés ²⁸
apoE-null	p27 ^{Kip1} ^{-/-} BMT in p27 ^{Kip1} ^{+/+} mice	Increase	Increase	Not studied	Díez-Juan <i>et al.</i> ²⁹
apoE-null	p27 ^{Kip1} T187A mutation	None	None	None	Sanz-González <i>et al.</i> ⁴⁰
apoE-null	p21 ^{Cip1} global inactivation	Reduction	None	Increase	Merched and Chan ⁴²
apoE-null	p21 ^{Cip1} ^{-/-} BMT in p21 ^{Cip1} ^{+/+} mice	Reduction	Not studied	Increase	Merched and Chan ⁴²
apoE-null	p21 ^{Cip1} global inactivation	Increase	Not studied	Not studied	Khanna ⁴⁴

BMT, bone marrow transplant.

Only studies reporting statistically significant effects are included.

apoE-null mice carrying an additional p53 allele that reproduces the normal expression and regulation of the endogenous p53 gene (apoE-null Super-p53 mice).²⁰ In this study, increased p53 function did not provoke significant differences in the size of atherosclerotic lesions within the ascending aorta, aortic arch, or thoracic aorta, irrespective of feeding with a high-fat diet or standard chow. Cell proliferation and apoptosis within the atheroma were similarly unaffected. These findings demonstrate that moderate p53 gain-of-function does not limit atheroma development in mice, and thus shed doubt on the utility of p53 activation as a means of preventing atherosclerosis. However, it remains possible that more intense increases in p53 function might have therapeutic potential.

2.2 Retinoblastoma protein

Rb plays a major role in cell proliferation and apoptosis.²¹ It is well established that hypophosphorylated Rb induces cell cycle arrest in G1 by two mechanisms: first, it impedes E2F-mediated transcription of genes involved in cell cycle progression (Figure 2), and secondly it actively represses transcription through the formation of the Rb-E2F transcription repressor complex. The role of Rb in the regulation of apoptosis was identified in loss-of-function studies, showing that Rb deficiency in mice triggers p53-dependent apoptosis²¹ and results in embryonic lethality.²²

The effects of Rb disruption on atherogenesis have been studied in apoE-null mice with selective inactivation of Rb in macrophages (macrophage-Rb^{del} apoE-null mice).²³ These animals develop larger and more advanced aortic atheromas than apoE-null mice, with a decreased macrophage content and an increased percentage of VSMCs. Apoptosis within the atheroma is almost undistinguishable between the two groups, indicating that macrophage Rb plays a minor role in cell-death within the atheroma. In contrast, the authors found a significant increase (2.6-fold) in lesional macrophage proliferation in macrophage-Rb^{del} apoE-null mice, suggesting that inhibition of macrophage proliferation is the major mechanism underlying the atheroprotective properties of Rb. Since Rb also modulates VSMC proliferation, senescence, and apoptosis,²⁴ future studies are warranted to investigate the effects on atherosclerosis development of selective Rb inactivation in VSMCs.

2.3 p27^{Kip1}

The Cip/Kip family member p27^{Kip1} is a CKI that plays a major role in the control of the mammalian cell cycle in various pathophysiological settings.²⁵ p27^{Kip1} is maximally translated and stable in quiescent cells, and contributes to growth arrest through the inhibition of cyclin-CDK complexes. Upon mitogenic stimulation, p27^{Kip1} is rapidly downregulated, allowing activation of cyclin E–

CDK2 and cyclin A–CDK2 complexes and the subsequent transcriptional activation of genes required for the G1/S transition and the initiation of DNA replication.^{25,26}

p27 acts as a tumour suppressor.²⁷ Mouse studies performed in our laboratory revealed that it also has atheroprotective functions. We first showed that genetic inactivation of p27^{Kip1} greatly accelerates diet-induced atherogenesis in apoE-null mice (~6-fold), coinciding with enhanced proliferation of macrophages and VSMCs within the atheroma (~4-fold).²⁸ Moreover, analysis of apoE-null mice with one p27^{Kip1} allele inactivated revealed that a moderate decrease in p27^{Kip1} protein expression is sufficient to hasten atherosclerosis development in this model. In a subsequent study, we demonstrated that haematopoietic cell-selective disruption of p27^{Kip1} in apoE-null mice enhances arterial macrophage proliferation and accelerates aortic atherosclerosis.²⁹ Interestingly, this strategy also augmented aortic expression of the proinflammatory cytokines CCL2/MCP-1 and CCL5/RANTES, suggesting that p27^{Kip1} might have proliferation-independent functions that affect the inflammatory response within the atheroma. In this regard, in addition to modulating proliferation, p27^{Kip1} regulates other cellular processes that may contribute to the atherosclerosis development, such as migration, apoptosis, and autophagy.^{30–37} Moreover, p27^{Kip1} interacts with a number of proteins that might influence atheroma development, such as the small GTPases RhoA³¹ and Rac³⁶ and the signalling adaptor Grb2.^{38,39} It remains to be demonstrated whether these proliferation-independent functions and/or interactions with signalling proteins contribute to the atheroprotective properties of p27^{Kip1}.

The molecular mechanisms that regulate p27 expression and function in the arterial wall remain ill defined. It is well established that p27^{Kip1} protein levels are mainly regulated by post-translational modifications. One of the most consistently demonstrated modifications of p27^{Kip1} is its phosphorylation at threonine 187 (T187), which is required for the degradation of p27^{Kip1} at the G1/S transition and in the G2 phase prior to mitosis.²⁶ To investigate whether this post-translational modification of p27^{Kip1} plays a role in atherosclerosis, our laboratory generated apoE-null mice with both p27^{Kip1} alleles replaced by a version carrying a T→A mutation at position 187 to block phosphorylation at this residue (apoE-null p27^{T187A} mice).⁴⁰ We found that aortic p27^{Kip1} expression was unaffected by the T187A mutation, and atheroma size, lesion cellularity, cell proliferation, and apoptosis were undistinguishable between fat-fed apoE-null p27^{T187A} and apoE-null mice. Hence, it can be concluded that phosphorylation of p27^{Kip1} at T187 is not implicated in the control of aortic p27^{Kip1} expression and atherosclerosis in hypercholesterolemic mice. Future studies are therefore warranted to analyse the effect of additional post-translational modifications of p27^{Kip1} on atherosclerosis development.

2.4 p21^{Cip1}

The Cip/Kip family member p21^{Cip1} is a major downstream target of p53 that modulates cell proliferation. Global inactivation of p21^{Cip1} reduces atherosclerosis burden in apoE-null mice fed either standard chow or a proatherogenic high-fat diet.^{41,42}

Moreover, transplantation of p21^{Cip1}-deficient bone marrow into apoE-null mice also reduces atheroma size, thus demonstrating a protective effect of p21^{Cip1}-deficiency in macrophages. This contrasts with studies on p53, p27^{Kip1}, and Rb, whose total or partial inactivation in mice aggravates atherosclerosis (see above). *In vivo* incorporation of bromodeoxyuridine (BrdU) into 20-week-old apoE-null mice fed a normal chow diet revealed no differences in lesional cell proliferation between p21^{Cip1}-deficient and p21^{Cip1}-wild-type mice, suggesting that the atherogenic actions of p21 are unrelated to its role as a cell cycle regulator. However, cell proliferation in the atherosclerotic plaque probably occurs at specific stages of lesion formation; so additional studies, with mice at different ages and fed with high fat for different periods, are required to precisely establish the consequences of p21^{Cip1} inactivation on lesional cell proliferation. Merched and Chan⁴² found significant upregulation of other growth suppressors upon inactivation of p21^{Cip1}, such as p16^{Ink4a}, Rb, and p53, and suggested that this might compensate the effect of p21^{Cip1} deficiency on cell proliferation. Specifically, the authors hypothesize that the increased levels of p16^{Ink4a}, a strong inhibitor of CDK4, would displace other Cip/Kip family members (p27^{Kip1} and p57^{Kip2}) from CDK4-cyclin D inhibitory complexes, making them available to inhibit CDK2 and thereby decelerating cell cycle progression and proliferation in the absence of p21^{Cip1}. Consistent with the previously reported antiapoptotic role of p21^{Cip1},⁴³ Merched and Chan⁴² found increased lesion apoptosis after both global and macrophage-specific inactivation of p21^{Cip1} (1.8- and 3-fold, respectively). Moreover, gene expression profiling of thioglycolate-elicited peritoneal macrophages from p21^{Cip1}-null mice revealed higher expression of putative atheroprotective factors (SR-BI, macrophage scavenger receptor A, and LDL-receptor related protein) and lower levels of proinflammatory molecules (macrophage inflammatory proteins 1 and 2 and interleukin-1α). p21^{Cip1}-deficient macrophages also exhibited a 2-fold increased phagocytic activity towards fluorescent latex microspheres and apoptotic thymocytes. In summary, Merched and Chan's⁴² work demonstrates that p21^{Cip1} is a proatherogenic molecule, since its inactivation limits the production of proinflammatory cytokines, enhances immune cell apoptosis, and upregulates phagocytic macrophage activity, which facilitates apoptotic cell clearance and prevents necrosis, thereby promoting plaque stability.

Merched and Chan's⁴² conclusions contrast with a very recent study that reports that inactivation of p21^{Cip1} renders mice more susceptible to high fat diet-induced atherosclerosis.⁴⁴ However, in this second study, the dietary fat level was only moderate, and the mice used were on a mixed genetic background (FVB), had low plasma lipid levels (maximum 100 mg/dL), and did not readily develop atherosclerosis. Moreover, atheroma development was analysed only in the coronary artery. In contrast, Merched and Chan⁴² validated their data through the use of two dietary regimens (regular chow and western diet), the study of global and macrophage-restricted ablation of p21^{Cip1} in a widely used mouse model of atherosclerosis (apoE-null mice), and analysis of atherosclerosis lesions in whole aortas and in cross-sections of the aortic sinus.

3. Cell cycle regulators in experimental graft atherosclerosis

Excessive cell proliferation and neointimal hyperplasia followed by atheromatous plaque development are common features of both vein graft and cardiac allograft atherosclerosis. In this section, we discuss different cell cycle-based strategies employed to inhibit experimental graft atherosclerosis (summarized in Table 2). We also discuss several animal studies that highlight the important role of the p53-p21^{Cip1} axis in this pathology.

3.1 Antisense oligodeoxynucleotides (ODNs) targeting cell cycle regulators

The use of complementary oligodeoxynucleotides (ODNs) to target CDK mRNAs and transcription factors involved in cell cycle regulation has been mainly investigated in experimental graft atherosclerosis. Mann *et al.*^{45,46} demonstrated the beneficial effects of selectively blocking the expression of the cell cycle regulators CDK1 (also called CDC2) and proliferating cell nuclear antigen (PCNA) in rabbit jugular veins grafted into carotid arteries. These engineered grafts underwent a phenotypic shift from hyperplasia towards hypertrophy, maintained a normal endothelial phenotype, and were resistant to diet-induced atherosclerosis. The benefits of this strategy have been confirmed by cotransfection of antisense ODNs to PCNA and CDK1 in a rat cardiac allograft model.⁴⁷ Moreover, transfection of antisense ODNs to PCNA alone significantly inhibits intimal hyperplasia in experimental rabbit vein grafts,⁴⁸ while transfection of antisense ODNs to CDK1 alone has protective effects in a mouse coronary allograft model.⁴⁹ Increased expression of CDK1 has been detected in the coronary arteries of chronically rejected allografts of non-human primates,⁵⁰ suggesting that this kinase is a key player in graft vasculopathies and a potential target for gene therapy. Another target of potential therapeutic interest is CDK2, since enhanced CDK2 mRNA expression has been found in the thickened intima of coronary arteries of mouse heterotopic cardiac allografts, and antisense CDK2 ODNs efficiently inhibit

neointima formation and VCAM-1 expression in this disease model.⁵¹

Antisense strategies have also targeted several 'immediate-early' genes implicated in vascular cell proliferation. Suggs *et al.*⁵² reported increased expression of c-fos and c-jun after perfusion of rat vein grafts, and showed that treatment of grafts with antisense ODNs to these 'immediate-early' genes significantly reduced the thickness of the intimal layer in the perianastomotic and midgraft regions. Similarly, expression of the c-myc oncoprotein has been correlated with VSMC proliferation during the period of maximal intimal thickening in rat experimental vein grafts,⁵³ and intraoperative application of antisense c-myc oligomers has been reported to inhibit neointima formation in a porcine vein graft model.^{54,55} Similar protective effects on intimal hyperplasia have been demonstrated for locally delivered antisense ODNs to the proto-oncogene c-myc in rabbit experimental vein grafts.⁵⁶

3.2 Decoy ODNs targeting cell cycle regulators

Other genetic approaches have used double-stranded ODNs encoding the consensus-binding sequences for transcription factors involved in the activation of atherogenic genes ('decoy oligonucleotides'). Treatment of experimental grafts with decoy ODNs for E2F has been extensively explored, and has provided strong evidence of reduced neointimal hyperplasia in mouse and non-human primate cardiac allografts^{57–59} and in rabbit^{60,61} and canine⁶² vein grafts. In these studies, E2F blockade correlated with VSMC growth arrest and suppression of positive cell cycle regulatory genes in the graft wall, and also with downregulation of inflammatory mediators such as NFkappaB⁵⁸ and E-selectin,⁵⁹ without inhibiting endothelial repopulation after vein bypass.⁶¹ Moreover, in the setting of diet-induced hypercholesterolaemia, delivery of E2F decoy ODNs provides vein grafts with long-term resistance to atherosclerosis.⁶⁰ Chimeric decoy ODNs, inhibiting both E2F and NFkappaB, reduced intimal hyperplasia and accelerated re-endothelialization in a rabbit model of vein graft, and these

Table 2 Reduction of neointimal thickening by antiproliferative gene therapy in animal models of graft vasculopathy

Strategy	Target gene	Animal model of graft atherosclerosis	Reference
Antisense ODNs	CDC2/PCNA	Rabbit vein graft	Mann <i>et al.</i> ^{45,46}
	CDC2/PCNA	Rat cardiac allograft	Miniati <i>et al.</i> ⁴⁷
	CDC2	Mouse cardiac allograft	Isobe <i>et al.</i> ⁴⁹
	PCNA	Rabbit vein graft	Fulton <i>et al.</i> ⁴⁸
	CDK2	Mouse cardiac allograft	Suzuki <i>et al.</i> ⁵¹
	c-fos/c-jun	Rat vein graft	Suggs <i>et al.</i> ⁵²
	c-myc	Porcine vein graft	Mannion <i>et al.</i> ^{54,55}
	c-myb	Rabbit vein graft	Fulton <i>et al.</i> ⁵⁶
'Decoy' ODNs	E2F	Mouse cardiac allograft	Kawauchi <i>et al.</i> ⁵⁷ and Suzuki <i>et al.</i> ⁵⁹
	E2F	Primate cardiac allograft	Kawauchi <i>et al.</i> ^{57,58}
	E2F	Rabbit vein graft	Ehsan <i>et al.</i> ^{60,61}
	E2F	Canine vein graft	Cho <i>et al.</i> ⁶²
	E2F/NFkappaB chimera	Rabbit vein graft	Miyake <i>et al.</i> ⁶³
Overexpression growth suppressors	p21 ^{Cip1}	Rabbit vein graft	Bai <i>et al.</i> ⁷⁰
	p53	Porcine vein graft	Wan <i>et al.</i> ⁶⁶

effects correlated with reduced accumulation of VSMCs and macrophages and with decreased expression of PDGF, VCAM-1, and MCP1.⁶³ Although it is beyond the scope of this review, it is worth noting that the E2F decoy strategy (edifoligide) has been explored in a series of clinical trials known as the PProject of Ex vivo Vein graft ENgineering via Transfection (PREVENT). While confirming the safety and viability of E2F decoy ODNs treatments, the four trials conducted to date have found that this treatment was clinically ineffective at preventing human graft failure (reviewed in⁶⁴).

3.3 The p53-p21^{Cip1} axis in experimental graft atherosclerosis

Several studies have highlighted the role of p53 and its target p21^{Cip1} in experimental graft atherosclerosis. Neointimal hyperplasia of vein grafts is significantly increased in p53-null mice, coinciding with significantly reduced apoptosis, and augmented and reduced lesional content of VSMCs and macrophages, respectively.⁶⁵ Moreover, cultured VSMCs exhibit increased proliferation and migration and decreased apoptosis in response to sodium nitroprusside, thus suggesting that p53 deficiency accelerates neointima formation by promoting VSMC proliferation and abrogating cell apoptosis.⁶⁵ In line with these findings, adenovirus-mediated p53 overexpression significantly upregulates apoptosis and reduces neointimal proliferation in porcine vein grafts, thereby limiting neointimal thickening.⁶⁶

In a rat model of vein arterialization, p21^{Cip1} is gradually down-regulated, reaching a minimum by day 7 that is sustained until day 90, suggesting that reduced p21^{Cip1} levels may contribute to vascular cell proliferation in this pathological model.⁶⁷ Although the same study revealed no changes in p27^{Kip1} and p16^{INK4a} expression, further studies are required in other graft models to conclusively address the role of CKIs in graft atherosclerosis, since regulation of these proteins appears to vary depending on the vascular bed.^{68,69} Interestingly, overexpression of p21^{Cip1} in rabbit vein grafts by intraoperative transfection not only significantly inhibits neointima formation but also induces maturation of VSMCs from the neonatal to the adult phenotype.⁷⁰ Additionally, oral administration of the antiallergic drug tranilast in murine cardiac transplantation models inhibits cardiac allograft vasculopathy in association with increased neointimal expression of p21^{Cip1} and decreased expression of PCNA.^{71,72}

4. Cell cycle regulators in human atherosclerosis

The importance of proliferation in the growth of atherosclerotic plaques has been extensively studied in human vascular obstructive lesions. In this section, we summarize several reports that analyse cell proliferation and the expression and localization of cell cycle regulators within human atherosclerotic plaques.

4.1 Cell proliferation in human atherosclerosis

The occurrence of cell proliferation in human vascular obstructive disease is demonstrated by the consistent finding of proliferation

markers in human primary atheromatous plaques and restenotic lesions.^{73–83} However, it should be noted that some studies have reported very low proliferation rates,^{73,74,76,78,80,82} while others find high proliferative activity^{75,81,83} in human atherosclerotic and restenotic lesions. Several factors may contribute to these conflicting findings, including technical issues (differences in tissue fixatives, antigen accessibility, analysis of different proliferation markers, etc.), differences in the arteries being analysed (for example, peripheral, coronary, and carotid arteries), and variation in the state of development of the atheroma (for example, fatty streaks, fibrolipid plaques, or complicated atheromas). Supporting this last possibility, complicated atheromas in human carotid artery samples (type VI) have recently been reported to exhibit higher proliferation rates than non-complicated fibrous lesions (type V).⁸⁴

Proliferating cells within human atherosclerotic plaques include VSMCs, leukocytes, and ECs. Rekhter and Gordon⁷⁸ demonstrated divergence in the proliferation rates of these different cell types in advanced atheromas from human carotid arteries. While monocyte/macrophages are the predominant proliferative cell type in the intima (46% monocyte/macrophages, vs. 9.7% α -actin immunoreactive VSMCs, 14.3% ECs, and 13.1% T-lymphocytes), proliferating VSMCs predominate in the media (44.4% VSMCs vs. 20% ECs, 13% monocyte/macrophages, and 14.3% T-lymphocytes). This study also revealed higher proliferation rates in the intimal lesion than in the underlying media (1.61 ± 0.35 vs. $0.05 \pm 0.03\%$, respectively), suggesting that different distributions of growth regulatory proteins and stimuli exist in different regions of human atherosclerotic arteries.

4.2 Expression and localization of cell cycle regulators in human atherosclerosis

As mentioned earlier, studies in animal models have demonstrated both atheroprotective and atherogenic actions for different cell cycle regulators. Human studies have demonstrated differential expression of these proteins in atherosclerotic lesions and healthy vessels,^{79–81,85–89} thus highlighting the role of cell cycle regulators in atherosclerosis development.

Quantitative immunoblotting analyses have demonstrated low p27^{Kip1} levels in human atherosclerotic and restenotic coronary arteries compared with aorta, internal mammary artery, and carotid artery thrombendarterectomy specimens.⁸⁵ In the same study, p21^{Cip1} was found to be upregulated in restenotic lesions compared with primary lesions and other vascular vessels. In another report, Tanner et al.⁷⁹ analysed CKI expression in healthy and atherosclerotic specimens of human coronary artery. Expression of p27^{Kip1} was abundant in non-proliferating cells within normal and atherosclerotic arteries, whereas p21^{Cip1} was undetectable in normal arteries but markedly upregulated in atherosclerotic tissue. Moreover, p27^{Kip1} and p21^{Cip1} expression was most frequently found in non-proliferating regions within the atheroma, suggesting an inverse correlation between arterial cell proliferation and CKI expression. Interestingly, p27^{Kip1} and TGF- β receptors are co-expressed in human atherosclerotic coronary artery specimens, suggesting that the anti-mitogenic action of TGF- β in these lesions might be mediated by p27^{Kip1}.⁸⁶

The transcriptional regulator p53 is overexpressed, but not mutated in human atherosclerotic tissue.⁸⁹ Moreover, Ihling *et al.*⁸⁷ reported that p53 and p21^{Cip1} are co-expressed in non-proliferating regions within advanced human carotid atherosclerotic plaques, thus indicating that p53-dependent transcriptional activation of p21^{Cip1} might protect against excessive vascular cell growth. The same group reported p53 expression in apoptotic cells within human atherosclerotic plaques.⁸⁸ Moreover, p53 and MDM2, a nuclear protein which promotes p53 degradation, co-localize in a few Ki67-positive cells, suggesting that the p53-dependent apoptosis or re-entry into the cell cycle might depend on the relative abundance of these two proteins.

In addition to their roles as inhibitors of cell proliferation, p16^{Ink4a}, p21^{Cip1}, and p53 might also play an important role in cellular senescence, a process relevant to advanced atherosclerosis.⁹⁰ Recent studies have revealed increased expression of p16^{Ink4a} and p21^{Cip1} in human coronary plaques, co-localizing with the activity of the senescence marker β -galactosidase (SA β G).⁹¹ Interestingly, cells from atherosclerotic lesions contain shorter telomeres,⁹¹ another characteristic linked to cell senescence and cardiovascular disease.⁹² Marfella *et al.*⁹³ also found that the expression of p16^{Ink4a} and p53 is markedly upregulated in atheromas from elderly patients compared with younger patients undergoing carotid endarterectomy.

4.3 Expression of cell cycle regulators in human graft atherosclerosis

As discussed earlier, animal studies highlight the role of the p53-p21^{Cip1} pathway in the pathophysiology of graft atherosclerosis. There is also evidence of p53 and p21^{Cip1} upregulation in human graft atherosclerosis. McLaren *et al.*⁹⁴ found elevated expression of p53 in cardiac allograft recipients with acute rejection, and suggested this oncosuppressor as a potential myocyte damage marker for the diagnosis of acute cardiac allograft rejection. Similarly, high levels of p53 were observed in atherosclerotic areas of human aorto-coronary saphenous vein bypass grafts, with a strong correlation between p53 expression and apoptosis (as determined by TUNEL staining) in the intima, but not in the media of vein grafts.⁹⁵ In contrast to these findings, Baas *et al.*⁹⁶ found almost undetectable p53 expression in coronary arteries from transplanted hearts, although its transcriptional target p21^{Cip1} was highly expressed.

5. Genetic evidence for the role of cell cycle regulators in human atherosclerosis

It is now widely accepted that the classic environmental risk factors only partly explain the development of atherosclerosis, and that genetic risk factors are critically involved in this pathology and its clinical manifestations. Genetic polymorphisms associated with atherosclerosis usually affect endothelial function, inflammation, lipid metabolism, or the thrombosis and fibrinolysis cascades. However, recent genetic studies have found robust genetic polymorphisms in genes involved in cell cycle regulation (mostly

CKIs), underlining the important role of cell proliferation in atherogenesis.

5.1 The 9p21 genetic polymorphisms and the INK4/ARF locus

The strongest and most replicated susceptibility locus for CAD and MI so far identified in humans is located on chromosome 9p21, and comprises several single nucleotide polymorphisms (SNPs) that have been consistently associated with these diseases in several independent Caucasian and Asian populations^{97–103} (and others, reviewed in¹⁰⁴), but not in African American subjects.^{97,105} This susceptibility locus is also associated with other vascular diseases, such as abdominal aortic and intracranial aneurysms.¹⁰⁶ The most replicated 9p21 SNPs associated with CAD and MI lie in a region 100 Kb centromeric to the INK4/ARF locus, thus suggesting a link between this locus and atherogenesis. The INK4/ARF locus plays essential roles in cell proliferation, apoptosis, and senescence, and encodes two CKIs (p15^{Ink4b} and p16^{Ink4a}), the p53-regulatory protein ARF, and a recently discovered non-coding RNA named ANRIL (for antisense noncoding RNA in the INK4 A locus). Strikingly, expression of all INK4/ARF transcripts is reduced in the peripheral blood T-cells of individuals homozygotic for a common 9p21 SNP associated with increased risk of atherosclerosis (rs10757278).¹⁰⁷ This provides a direct link between these atherosclerosis-associated SNPs and the INK4/ARF locus and suggests a key role for p15^{Ink4b}, p16^{Ink4a}, ARF, and ANRIL in protecting against atherosclerosis development. Studies with genetically modified mice are warranted to assess whether INK4/ARF gene products indeed affect atherogenesis.

5.2 Genetic polymorphisms in CKIs of the Cip/Kip family

Several genetic studies have identified MI-associated variants in human genes encoding CKIs of the Cip/Kip family. For example, CDKN1C, the gene encoding p57^{Kip2}, contains two polymorphisms (a promoter GT-repeat and a variable number of repeats of the amino acid PAPA-motif) that were found to be associated with MI.¹⁰⁸ Similarly, the -838C > A SNP within the CDKN1B gene (encoding p27^{Kip1}) was found to be associated with increased risk of MI and with reduced basal p27^{Kip1} promoter activity in proliferating human cells.¹⁰⁹ However, this SNP has also recently been reported to confer a decreased risk of in-stent restenosis and a 20-fold increase in basal p27^{Kip1} promoter activity in quiescent human cells.¹¹⁰ This apparent discrepancy, between decreased risk of in-stent restenosis and increased risk of MI, might be explained by the divergent pathophysiological roles of VSMCs in restenotic and atherosclerotic lesions. In in-stent restenosis, decreased VSMC proliferation can reduce neointima formation, but in atherosclerotic plaques it can also diminish fibrous cap thickness, increasing plaque vulnerability and potentially accounting for the increased risk of MI. Regarding the divergent effects of this SNP on p27^{Kip1} promoter activity, it should be noted that this activity was assayed in different cell types (Jurkat vs. HEK293 cells) and in different culture conditions (proliferating vs. quiescent cells). Further studies are therefore required to conclusively define the consequences of the -838C > A SNP on p27^{Kip1} expression.

Moreover, larger cohorts need to be analysed to validate these genotype-disease associations.

6. Concluding remarks

Mounting evidence demonstrates that excessive cell proliferation is an essential hallmark of atherosclerosis development, both in animal models and in human beings. Therefore, proteins involved in the regulation of cell proliferation in the vascular wall are receiving special attention as potential therapeutic targets for the treatment of vascular diseases. Studies in genetically engineered mice have conclusively demonstrated the atheroprotective properties of the growth suppressors p53, p27^{Kip1}, and Rb and the surprising proatherogenic function of p21^{Cip1}, despite its well-defined cytostatic activity. Interestingly, apart from their roles in regulating cell proliferation, these proteins also appear to modulate other cellular processes within the atheroma, such as apoptosis and cell senescence. Further animal studies are warranted to analyse the role of other cell cycle regulators in the development of atherosclerosis, especially in light of the genetic evidence suggesting important roles for CKIs and ARF in the development of CAD and MI in humans.

Although the evidence is conclusive that altering the cell cycle machinery affects atherosclerosis development in animal models, we are unaware of clinical trials targeting cell cycle regulators for the treatment of native atherosclerosis. However, given that administration of rapamycin (sirolimus) alleviates atherosclerosis in the mouse (reviewed in¹¹), prospective long-term trials should be instigated to assess whether renal and heart transplant recipients treated with this immunosuppressive and antiproliferative drug exhibit a reduction in morbidity and mortality attributable to cardiovascular disease. In the case of graft atherosclerosis, results obtained from animal studies have already been translated into therapeutic strategies, although so far these have proved to be unsuccessful in humans. Therefore, as our knowledge of the role of cell cycle regulators in atherosclerosis grows, the challenge will be to translate all this information into valuable diagnostic and therapeutic tools for humans, as has been achieved in the setting of post-angioplasty restenosis with the use of stents engineered to deliver cytostatic drugs.

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References

- Vidal A, Koff A. Cell cycle inhibitors: three families united by a common cause. *Gene* 2000;**247**:1–15.
- Chisolm GM, Chai Y-C. Regulation of cell growth by oxidized LDL. *Free Radic Biol Med* 2000;**28**:1697–1707.
- Lamharzi N, Renard CB, Kramer F, Pennathur S, Heinecke JW, Chait A et al. Hyperlipidemia in concert with hyperglycemia stimulates the proliferation of macrophages in atherosclerotic lesions. *Diabetes* 2004;**53**:3217–3225.
- Liu YJ, Saini A, Cohen DJ, Ooi BS. Modulation of macrophage proliferation by hyperglycemia. *Mol Cell Endocrinol* 1995;**114**:187–192.
- Zettler ME, Prociuk MA, Austria JA, Massaeli H, Zhong G, Pierce GN. OxLDL stimulates cell proliferation through a general induction of cell cycle proteins. *Am J Physiol Heart Circ Physiol* 2003;**284**:H644–H653.
- Tsai JC, Wang H, Perrella MA, Yoshizumi M, Sibinga NE, Tan LC et al. Induction of cyclin A gene expression by homocysteine in vascular smooth muscle cells. *J Clin Invest* 1996;**97**:146–153.
- Diep QN, El Mabrouk M, Touyz RM, Schiffrin EL. Expression of cell cycle proteins in blood vessels of angiotensin II-infused rats: role of AT1 receptors. *Hypertension* 2001;**37**:604–608.
- Andrés V. Control of vascular cell proliferation and migration by cyclin-dependent kinase signalling: new perspectives and therapeutic potential. *Cardiovasc Res* 2004;**63**:11–21.
- Dzau VJ, Braun-Dullaeus RC, Sedding DG. Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. *Nat Med* 2002;**8**:1249–1256.
- Fuster JJ, Sanz-González SM, Moll UM, Andrés V. Classic and novel roles of p53: prospects for anticancer therapy. *Trends Mol Med* 2007;**13**:192–199.
- Guevara NV, Kim HS, Antonova EI, Chan L. The absence of p53 accelerates atherosclerosis by increasing cell proliferation in vivo. *Nat Med* 1999;**5**:335–339.
- Mercer J, Figg N, Stoneman V, Braganza D, Bennett MR. Endogenous p53 protects vascular smooth muscle cells from apoptosis and reduces atherosclerosis in ApoE knockout mice. *Circ Res* 2005;**96**:667–674.
- van Vlijmen BJ, Gerritsen G, Franken AL, Boesten LS, Kockx MM, Gijbels MJ et al. Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. *Circ Res* 2001;**88**:780–786.
- Merched AJ, Williams E, Chan L. Macrophage-specific p53 expression plays a crucial role in atherosclerosis development and plaque remodeling. *Arterioscler Thromb Vasc Biol* 2003;**23**:1608–1614.
- Boesten LS, Zadelaar AS, van Nieuwkoop A, Hu L, Teunisse AF, Jochemsen AG et al. Macrophage p53 controls macrophage death in atherosclerotic lesions of apolipoprotein E deficient mice. *Atherosclerosis* 2009; in press.
- von der Thüsen JH, van Vlijmen BJ, Hoebe RC, Kockx MM, Havekes LM, van Berkel TJ et al. Induction of atherosclerotic plaque rupture in apolipoprotein E-/- mice after adenovirus-mediated transfer of p53. *Circulation* 2002;**105**:2064–2070.
- Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;**275**:964–967.
- Sata M, Saiura A, Kunisato A, Tojo A, Okada S, Tokuhisa T et al. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med* 2002;**8**:403–409.
- Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A et al. Evidence for circulating bone marrow-derived endothelial cells. *Blood* 1998;**92**:362–367.
- Sanz-González SM, Barquín L, García-Cao I, Roque M, González JM, Fuster JJ et al. Increased p53 gene dosage reduces neointimal thickening induced by mechanical injury but has no effect on native atherosclerosis. *Cardiovasc Res* 2007;**75**:803–812.
- Harbour JW, Dean DC. Rb function in cell cycle regulation and apoptosis. *Nat Cell Biol* 2000;**2**:E65–E67.
- Clarke AR, Maandag ER, van Roon M, van der Lugt NM, van der Valk M, Hooper ML et al. Requirement for a functional Rb-1 gene in murine development. *Nature* 1992;**359**:328–330.
- Boesten LS, Zadelaar AS, van Nieuwkoop A, Hu L, Jonkers J, van de Water B et al. Macrophage retinoblastoma deficiency leads to enhanced atherosclerosis development in ApoE-deficient mice. *FASEB J* 2006;**20**:953–955.
- Bennett MR, Macdonald K, Chan SV, Boyle JJ, Weissberg PL. Cooperative interactions between RB and p53 regulate cell proliferation, cell senescence, and apoptosis in human vascular smooth muscle cells from atherosclerotic plaques. *Circ Res* 1998;**82**:704–712.
- Slingerland J, Pagano M. Regulation of the cdk inhibitor p27 and its deregulation in cancer. *J Cell Physiol* 2000;**183**:10–17.
- Borriello A, Cucciolla V, Oliva A, Zappia V, Della Ragione F. p27Kip1 metabolism: a fascinating labyrinth. *Cell Cycle* 2007;**6**:1053–1061.
- Fero ML, Randel E, Gurley KE, Roberts JM, Kemp CJ. The murine gene p27Kip1 is haplo-insufficient for tumour suppression. *Nature* 1998;**396**:177–180.

28. Díez-Juan A, Andrés V. The growth suppressor p27(Kip1) protects against diet-induced atherosclerosis. *FASEB J* 2001;**15**:1989–1995.
29. Díez-Juan A, Pérez P, Aracil M, Sancho D, Bernad A, Sánchez-Madrid F et al. Selective inactivation of p27(Kip1) in hematopoietic progenitor cells increases neointimal macrophage proliferation and accelerates atherosclerosis. *Blood* 2004;**103**:158–161.
30. Baldassarre G, Belletti B, Nicoloso MS, Schiappacassi M, Vecchione A, Spessotto P et al. p27(Kip1)-stathmin interaction influences sarcoma cell migration and invasion. *Cancer Cell* 2005;**7**:51–63.
31. Besson A, Gurian-West M, Schmidt A, Hall A, Roberts JM. p27Kip1 modulates cell migration through the regulation of RhoA activation. *Genes Dev* 2004;**18**:862–876.
32. Chen Q, Xie W, Kuhn DJ, Voorhees PM, Lopez-Girona A, Mendy D et al. Targeting the p27 E3 ligase SCF(Skp2) results in p27- and Skp2-mediated cell cycle arrest and activation of autophagy. *Blood* 2008;**111**:4690–4699.
33. Díez-Juan A, Andrés V. Coordinate control of proliferation and migration by the p27Kip1/cyclin-dependent kinase/retinoblastoma pathway in vascular smooth muscle cells and fibroblasts. *Circ Res* 2003;**92**:402–410.
34. Hiromura K, Pippin JW, Fero ML, Roberts JM, Shankland SJ. Modulation of apoptosis by the cyclin-dependent kinase inhibitor p27Kip1. *J Clin Invest* 1999;**103**:597–604.
35. Liang J, Shao SH, Xu ZX, Hennessy B, Ding Z, Larrea M et al. The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. *Nat Cell Biol* 2007;**9**:218–224.
36. McAllister SS, Becker-Hapak M, Pintucci G, Pagano M, Dowdy SF. Novel p27(kip1) C-terminal scatter domain mediates Rac-dependent cell migration independent of cell cycle arrest functions. *Mol Cell Biol* 2003;**23**:216–228.
37. Goukassian D, Díez-Juan A, Asahara T, Schratzberger P, Silver M, Murayama T et al. Overexpression of p27(Kip1) by doxycycline-regulated adenoviral vectors inhibits endothelial cell proliferation and migration and impairs angiogenesis. *FASEB J* 2001;**15**:1877–1885.
38. Sugiyama Y, Tomoda K, Tanaka T, Arata Y, Yoneda-Kato N, Kato J-Y. Direct binding of the signal-transducing adaptor Grb2 facilitates down-regulation of the cyclin-dependent kinase inhibitor p27Kip1. *J Biol Chem* 2001;**276**:12084–12090.
39. Moeller SJ, Head ED, Sheaff RJ. p27Kip1 inhibition of GRB2-SOS formation can regulate Ras activation. *Mol Cell Biol* 2003;**23**:3735–3752.
40. Sanz-González SM, Melero-Fernandez de Mera R, Malek NP, Andrés V. Atheroma development in apolipoprotein E-null mice is not regulated by phosphorylation of p27(Kip1) on threonine 187. *J Cell Biochem* 2006;**97**:735–743.
41. Andrés V. Unexpected proatherogenic properties of p21: beyond cell cycle control? *Circulation* 2004;**110**:3749–3752.
42. Merched AJ, Chan L. Absence of p21Waf1/Cip1/Sdi1 modulates macrophage differentiation and inflammatory response and protects against atherosclerosis. *Circulation* 2004;**110**:3830–3841.
43. Gartel AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Mol Cancer Ther* 2002;**1**:639–649.
44. Khanna AK. Enhanced susceptibility of cyclin kinase inhibitor p21 knockout mice to high fat diet induced atherosclerosis. *J Biomed Sci* 2009;**16**:66–78.
45. Mann MJ, Gibbons GH, Kernoff RS, Diet FP, Tsao PS, Cooke JP et al. Genetic engineering of vein grafts resistant to atherosclerosis. *Proc Natl Acad Sci USA* 1995;**92**:4502–4506.
46. Mann MJ, Gibbons GH, Tsao PS, von der Leyen HE, Cooke JP, Buitrago R et al. Cell cycle inhibition preserves endothelial function in genetically engineered rabbit vein grafts. *J Clin Invest* 1997;**99**:1295–1301.
47. Miniati DN, Hoyt EG, Feeley BT, Poston RS, Robbins RC. Ex vivo antisense oligonucleotides to proliferating cell nuclear antigen and Cdc2 kinase inhibit graft coronary artery disease. *Circulation* 2000;**102**:III237–III242.
48. Fulton GJ, Davies MG, Barber L, Svendsen E, Hagen PO. Locally applied antisense oligonucleotide to proliferating cell nuclear antigen inhibits intimal thickening in experimental vein grafts. *Ann Vasc Surg* 1998;**12**:412–417.
49. Isobe M, Suzuki J, Morishita R, Kaneda Y, Amano J. Gene therapy for heart transplantation-associated coronary arteriosclerosis. *Ann N Y Acad Sci* 2000;**902**:77–83.
50. Schoenbeck A, Isobe M, Suzuki J, Kato M, Kitazawa N, Amano J et al. Expression of cell division cycle 2 kinase transcription in chronically rejected cardiac allografts of nonhuman primates. *Heart Vessels* 1997;**12**:275–279.
51. Suzuki J, Isobe M, Morishita R, Aoki M, Horie S, Okubo Y et al. Prevention of graft coronary arteriosclerosis by antisense cdk2 kinase oligonucleotide. *Nat Med* 1997;**3**:900–903.
52. Suggs WD, Olson SC, Madnani D, Patel S, Veith FJ. Antisense oligonucleotides to c-fos and c-jun inhibit intimal thickening in a rat vein graft model. *Surgery* 1999;**126**:443–449.
53. Ramírez JA, Sánchez LA, Marín ML, Lyon RT, Parsons RE, Suggs WD et al. c-MYC oncoprotein production in experimental vein graft intimal hyperplasia. *J Surg Res* 1996;**61**:323–329.
54. Mannon JD, Ormont ML, Magno MG, O'Brien JE, Shi Y, Zalewski A. Sustained reduction of neointima with c-myc antisense oligonucleotides in saphenous vein grafts. *Ann Thorac Surg* 1998;**66**:1948–1952.
55. Mannon JD, Ormont ML, Shi Y, O'Brien JE Jr, Chung W, Roque F et al. Saphenous vein graft protection: effects of c-myc antisense. *J Thorac Cardiovasc Surg* 1998;**115**:152–161.
56. Fulton GJ, Davies MG, Koch WJ, Dalen H, Svendsen E, Hagen PO. Antisense oligonucleotide to proto-oncogene c-myc inhibits the formation of intimal hyperplasia in experimental vein grafts. *J Vasc Surg* 1997;**25**:453–463.
57. Kawauchi M, Suzuki J, Morishita R, Wada Y, Izawa A, Tomita N et al. Gene therapy for attenuating cardiac allograft arteriopathy using ex vivo E2F decoy transfection by HVJ-AVE-liposome method in mice and nonhuman primates. *Circ Res* 2000;**87**:1063–1068.
58. Kawauchi M, Suzuki J, Wada Y, Morishita R, Kaneda Y, Isobe M et al. Downregulation of nuclear factor kappa B expression in primate cardiac allograft arteries after E2F decoy transfection. *Transplant Proc* 2001;**33**:451.
59. Suzuki J, Isobe M, Morishita R, Izawa A, Yamazaki S, Okubo Y et al. E2F decoy suppresses E-selectin expression in murine cardiac allograft arteriopathy. *Transplant Proc* 1999;**31**:2018–2019.
60. Ehsan A, Mann MJ, Dell'Acqua G, Dzau VJ. Long-term stabilization of vein graft wall architecture and prolonged resistance to experimental atherosclerosis after E2F decoy oligonucleotide gene therapy. *J Thorac Cardiovasc Surg* 2001;**121**:714–722.
61. Ehsan A, Mann MJ, Dell'Acqua G, Tamura K, Braun-Dullaeus R, Dzau VJ. Endothelial healing in vein grafts: proliferative burst unimpaired by genetic therapy of neointimal disease. *Circulation* 2002;**105**:1686–1692.
62. Cho WH, Lee SO, Kim HT, Ahn JD, Lee IK. E2F decoy oligodeoxynucleotides on neointimal hyperplasia in canine vein graft. *Transplant Proc* 2005;**37**:77–79.
63. Miyake T, Aoki M, Morishita R. Inhibition of anastomotic intimal hyperplasia using a chimeric decoy strategy against NFkappaB and E2F in a rabbit model. *Cardiovasc Res* 2008;**79**:706–714.
64. Hoel AW, Conte MS. Edifoligide: a transcription factor decoy to modulate smooth muscle cell proliferation in vein bypass. *Cardiovasc Drug Rev* 2007;**25**:221–234.
65. Mayr U, Mayr M, Li C, Wernig F, Dietrich H, Hu Y et al. Loss of p53 accelerates neointimal lesions of vein bypass grafts in mice. *Circ Res* 2002;**90**:197–204.
66. Wan S, George SJ, Nicklin SA, Yim AP, Baker AH. Overexpression of p53 increases lumen size and blocks neointima formation in porcine interposition vein grafts. *Mol Ther* 2004;**9**:689–698.
67. Borin TF, Miyakawa AA, Cardoso L, de Figueiredo Borges L, Gonçalves GA, Krieger JE. Apoptosis, cell proliferation and modulation of cyclin-dependent kinase inhibitor p21(cip1) in vascular remodelling during vein arterialization in the rat. *Int J Exp Pathol* 2009;**90**:328–337.
68. Castro C, Díez-Juan A, Cortés MJ, Andrés V. Distinct regulation of mitogen-activated protein kinases and p27Kip1 in smooth muscle cells from different vascular beds. *J Biol Chem* 2003;**278**:4482–4490.
69. Yang Z, Oemar BS, Carrel T, Kipfer B, Julmy F, Luscher TF. Different proliferative properties of smooth muscle cells of human arterial and venous bypass vessels: role of PDGF receptors, mitogen-activated protein kinase, and cyclin-dependent kinase inhibitors. *Circulation* 1998;**97**:181–187.
70. Bai H, Morishita R, Kida I, Yamakawa T, Zhang W, Aoki M et al. Inhibition of intimal hyperplasia after vein grafting by in vivo transfer of human senescent cell-derived inhibitor-1 gene. *Gene Ther* 1998;**5**:761–769.
71. Izawa A, Suzuki J, Takahashi W, Amano J, Isobe M. Tranilast inhibits cardiac allograft vasculopathy in association with p21(Waf1/Cip1) expression on neointimal cells in murine cardiac transplantation model. *Arterioscler Thromb Vasc Biol* 2001;**21**:1172–1178.
72. Saiura A, Sata M, Hirata Y, Nagai R, Makuuchi M. Tranilast inhibits transplant-associated coronary arteriosclerosis in a murine model of cardiac transplantation. *Eur J Pharmacol* 2001;**433**:163–168.
73. Gordon D, Reidy MA, Benditt EP, Schwartz SM. Cell proliferation in human coronary arteries. *Proc Natl Acad Sci USA* 1990;**87**:4600–4604.
74. Katsuda S, Coltrera MD, Ross R, Gown AM. Human atherosclerosis. IV. Immunocytochemical analysis of cell activation and proliferation in lesions of young adults. *Am J Pathol* 1993;**142**:1787–1793.
75. Kearney M, Pieczek A, Haley L, Losordo DW, Andrés V, Schainfeld R et al. Histopathology of in-stent restenosis in patients with peripheral artery disease. *Circulation* 1997;**95**:1998–2002.
76. O'Brien ER, Alpers CE, Stewart DK, Ferguson M, Tran N, Gordon D et al. Proliferation in primary and restenotic coronary atherectomy tissue. Implications for antiproliferative therapy. *Circ Res* 1993;**73**:223–231.

77. Orekhov AN, Andreeva ER, Mikhailova IA, Gordon D. Cell proliferation in normal and atherosclerotic human aorta: proliferative splash in lipid-rich lesions. *Atherosclerosis* 1998;**139**:41–48.
78. Rekhter MD, Gordon D. Active proliferation of different cell types, including lymphocytes, in human atherosclerotic plaques. *Am J Pathol* 1995;**147**:668–677.
79. Tanner FC, Yang ZY, Duckers E, Gordon D, Nabel GJ, Nabel EG. Expression of cyclin-dependent kinase inhibitors in vascular disease. *Circ Res* 1998;**82**:396–403.
80. Veinot JP, Ma X, Jelley J, O'Brien ER. Preliminary clinical experience with the pull-back atherectomy catheter and the study of proliferation in coronary plaques. *Can J Cardiol* 1998;**14**:1457–1463.
81. Wei GL, Krasinski K, Kearney M, Isner JM, Walsh K, Andrés V. Temporally and spatially coordinated expression of cell cycle regulatory factors after angioplasty. *Circ Res* 1997;**80**:418–426.
82. O'Brien ER, Urieli-Shoval S, Garvin MR, Stewart DK, Hinohara T, Simpson JB et al. Replication in restenotic atherectomy tissue. *Atherosclerosis* 2000;**152**:117–126.
83. Pickering JG, Weir L, Jekanowski J, Kearney MA, Isner JM. Proliferative activity in peripheral and coronary atherosclerotic plaque among patients undergoing percutaneous revascularization. *J Clin Invest* 1993;**91**:1469–1480.
84. Manolakou P, Angelopoulou R, Bakoyiannis C, Psathas E, Bastounis E, Kavantzis N et al. Cellular proliferation in complicated versus uncomplicated atherosclerotic lesions: total cell population, foam cells and newly formed microvessels. *Tissue Cell* 2009;**41**:408–413.
85. Braun-Dullaues RC, Ziegler A, Bohle RM, Bauer E, Hein S, Tillmanns H et al. Quantification of the cell cycle inhibitors p27(Kip1) and p21(Cip1) in human atherectomy specimens: primary stenosis versus restenosis. *J Lab Clin Med* 2003;**141**:179–189.
86. Ihling C, Technau K, Gross V, Schulte-Monting J, Zeiher AM, Schaefer HE. Concordant upregulation of type II-TGF-beta-receptor, the cyclin-dependent kinases inhibitor p27Kip1 and cyclin E in human atherosclerotic tissue: implications for lesion cellularity. *Atherosclerosis* 1999;**144**:7–14.
87. Ihling C, Menzel G, Wellens E, Monting JS, Schaefer HE, Zeiher AM. Topographical association between the cyclin-dependent kinases inhibitor P21, p53 accumulation, and cellular proliferation in human atherosclerotic tissue. *Arterioscler Thromb Vasc Biol* 1997;**17**:2218–2224.
88. Ihling C, Haendeler J, Menzel G, Hess RD, Fraedrich G, Schaefer HE et al. Co-expression of p53 and MDM2 in human atherosclerosis: implications for the regulation of cellularity of atherosclerotic lesions. *J Pathol* 1998;**185**:303–312.
89. Iacopetta B, Wysocki S, Norman P, House A. The p53 tumor-suppressor gene is overexpressed but not mutated in human atherosclerotic tissue. *Int J Oncol* 1995;**7**:399–402.
90. Minamino T, Komuro I. Vascular cell senescence: Contribution to atherosclerosis. *Circ Res* 2007;**100**:15–26.
91. Matthews C, Gorenne I, Scott S, Figg N, Kirkpatrick P, Ritchie A et al. Vascular smooth muscle cells undergo telomere-based senescence in human atherosclerosis: effects of telomerase and oxidative stress. *Circ Res* 2006;**99**:156–164.
92. Fuster JJ, Andrés V. Telomere biology and cardiovascular disease. *Circ Res* 2006;**99**:1167–1180.
93. Marfella R, Di Filippo C, Laieta MT, Vestini R, Barbieri M, Sangiulo P et al. Effects of ubiquitin-proteasome system deregulation on the vascular senescence and atherosclerosis process in elderly patients. *J Gerontol A Biol Sci Med Sci* 2008;**63**:200–203.
94. McLaren BK, Venkatesh PK, Misra P, Zhang PL, Fowler MR. Increased expression of p53 protein correlates with the extent of myocyte damage in cardiac allograft rejection. *Congest Heart Fail* 2008;**14**:293–297.
95. Wang AY, Bobryshev YV, Cherian SM, Liang H, Tran D, Inder SJ et al. Expression of apoptosis-related proteins and structural features of cell death in explanted aortocoronary saphenous vein bypass grafts. *Cardiovasc Surg* 2001;**9**:319–328.
96. Baas IO, Offerhaus JA, El-Deiry WS, Wu TC, Hutchins GM, Kasper EK et al. The WAF1-mediated p53 growth-suppressor pathway is intact in the coronary arteries of heart transplant recipients. *Hum Pathol* 1996;**27**:324–329.
97. McPherson R, Pertsemidis A, Kavaslar N, Stewart A, Roberts R, Cox DR et al. A common allele on chromosome 9 associated with coronary heart disease. *Science* 2007;**316**:1488–1491.
98. Helgadóttir A, Thorleifsson G, Manolescu A, Gretarsdóttir S, Blondal T, Jonasdóttir A et al. A common variant on chromosome 9p21 affects the risk of myocardial infarction. *Science* 2007;**316**:1491–1493.
99. Samani NJ, Erdmann J, Hall AS, Hengstenberg C, Mangino M, Mayer B et al. Genome wide association analysis of coronary artery disease. *N Engl J Med* 2007;**357**:443–453.
100. Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 2007;**447**:661–678.
101. Schunkert H, Gotz A, Braund P, McGinnis R, Tregouet DA, Mangino M et al. Repeated replication and a prospective meta-analysis of the association between chromosome 9p21.3 and coronary artery disease. *Circulation* 2008;**117**:1675–1684.
102. Broadbent HM, Peden JF, Lorkowski S, Goel A, Ongen H, Green F et al. Susceptibility to coronary artery disease and diabetes is encoded by distinct, tightly linked SNPs in the ANRIL locus on chromosome 9p. *Hum Mol Genet* 2008;**17**:806–814.
103. Shen GQ, Li L, Rao S, Abdullah KG, Ban JM, Lee BS et al. Four SNPs on chromosome 9p21 in a South Korean population implicate a genetic locus that confers high cross-race risk for development of coronary artery disease. *Arterioscler Thromb Vasc Biol* 2008;**28**:360–365.
104. Tousoulis D, Briasoulis A, Papageorgiou N, Antoniadis C, Stefanadis C. Candidate gene polymorphisms and the 9p21 locus in acute coronary syndromes. *Trends Mol Med* 2008;**14**:441–449.
105. Assimes TL, Knowles JW, Basu A, Iribarren C, Southwick A, Tang H et al. Susceptibility locus for clinical and subclinical coronary artery disease at chromosome 9p21 in the multi-ethnic ADVANCE study. *Hum Mol Genet* 2008;**17**:2320–2328.
106. Helgadóttir A, Thorleifsson G, Magnusson KP, Gretarsdóttir S, Steinthorsdóttir V, Manolescu A et al. The same sequence variant on 9p21 associates with myocardial infarction, abdominal aortic aneurysm and intracranial aneurysm. *Nat Genet* 2008;**40**:217–224.
107. Liu Y, Sanoff HK, Cho H, Burd CE, Torrice C, Mohlke KL et al. INK4/ARF transcript expression is associated with chromosome 9p21 variants linked to atherosclerosis. *PLoS One* 2009;**4**:e5027.
108. Rodriguez I, Coto E, Reguero JR, Gonzalez P, Andres V, Lozano I et al. Role of the CDKN1A/p21, CDKN1C/p57, and CDKN2A/p16 genes in the risk of atherosclerosis and myocardial infarction. *Cell Cycle* 2007;**6**:620–625.
109. González P, Díez-Juan A, Coto E, Álvarez V, Reguero JR, Batalla A et al. A single-nucleotide polymorphism in the human p27kip1 gene (-838C > A) affects basal promoter activity and the risk of myocardial infarction. *BMC Biol* 2004;**2**:5–10.
110. van Tiel CM, Bonta PI, Rittersma SZ, Beijl MA, Bradley EJ, Klous AM et al. p27kip1-838C > A single nucleotide polymorphism is associated with restenosis risk after coronary stenting and modulates p27kip1 promoter activity. *Circulation* 2009;**120**:669–676.
111. Andrés V, Castro C, Campistol JM. Potential role of proliferation signal inhibitors on atherosclerosis in renal transplant patients. *Nephrol Dial Transplant* 2006;**21**:iii14–iii17.